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Transient receptor potential melastatin-3 (TRPM3)-induced activation of AP-1 requires Ca^{2+} ions and the transcription factors c-Jun, ATF2, and TCF

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Running title: TRPM3-triggered AP-1 activation

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Abbreviations:

AP-1, activator protein-1; bZIP, basic region leucine zipper; MAP kinase, mitogen activated protein kinase; PKC, protein kinase C; SRE, serum response element; SRF, serum response factor; TRE, 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-responsive element; TRP, transient receptor potential

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Abstract

The steroid pregnenolone sulfate activates the transcription factor activator protein-1 (AP-1) via stimulation of transient receptor potential melastatin-3 (TRPM3) channels. Here, we show that the signaling pathway requires an influx of Ca^{2+} ions into the cells and a rise in the intracellular Ca^{2+} levels. The upregulation of AP-1 was attenuated in cells that overexpressed MAP kinase phosphatase (MKP)-1, indicating that Ca^{2+} ions prolong the signaling cascade via activation of MAP kinases. On the transcriptional level, expression of a dominant-negative mutant of the basic region leucine zipper (bZIP) protein c-Jun, a major constituent of the AP-1 transcription factor complex, or expression of a c-Jun-specific small-hairpin (sh) RNA attenuated pregnenolone sulfate-induced AP-1 activation. In addition, stimulation of TRPM3 channels increased the transcriptional activation potential of the bZIP protein ATF2. Inhibition of ATF2 target gene expression via expression of a dominant-negative mutant of ATF2 or expression of an ATF2-specific shRNA interfered with TRPM3-mediated stimulation of AP-1. Moreover, we show that a dominant-negative mutant of the ternary complex factor (TCF) Elk-1 attenuated the upregulation of AP-1 following stimulation of TRPM3 channels. Thus, c-Jun, ATF2 and TCFs are required to connect the intracellular signaling cascade elicited by activation of TRPM3 channels with enhanced transcription of AP-1 regulated genes. We conclude that pregnenolone sulfate-induced TRPM3 channel activation changes the gene expression pattern of the cells by activating transcription of c-Jun, ATF2 and TCF controlled genes.

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Introduction

Transient receptor potential melastatin-3 (TRPM3) is a typical TRP channel, containing six transmembrane domains, a pore domain between the fifth and sixth transmembrane domains and a cytosolic location of both the aminoterminus and the carboxyterminus (Thiel et al., 2013). TRPM3 channels are expressed in various tissues, including kidney, liver, ovary, brain, spinal cord, pituitary gland, vascular smooth muscle, testis and β-cells of the pancreas (Grimm et al., 2003; Lee et al., 2003; Oberwinkler et al., 2005; Wagner et al., 2008; Naylor et al., 2010).

Several metabolites were described to function as activators of TRPM3 channels, including the steroid pregnenolone sulfate, the lipid D-erythro-sphingosine, and the L-type voltage-gated Ca^{2+} channel inhibitor nifedipine (Grimm et al., 2003; Wagner et al., 2008; Naylor et al., 2010; Islam, 2011). A comprehensive analysis of different compounds revealed that pregnenolone sulfate is currently the most powerful known activator of TRPM3 (Lesch et al., 2014). However, the concentration of pregnenolone sulfate required to stimulate TRPM3 channels is in the micromolar range, suggesting that pregnenolone sulfate is not a physiological agonist of TRPM3 and may have only pharmacological relevance.

The analysis of TRPM3-deficient mice revealed that TRPM3 is a nociceptor channel involved in the detection of noxious heat in the somatosensory system (Vriens et al., 2011). The mutant mice exhibited deficiencies in acute heat sensing and inflammatory heat hyperalgesia. It has also been proposed that TRPM3 functions as an ionotropic steroid receptor in pancreatic β-cells responsible for modulating insulin biosynthesis and secretion (Wagner et al., 2008). However, TRPM3-deficient mice did not show alterations in resting blood glucose levels (Vriens et al., 2011). Thus, TRPM3 may play no or only a marginal role in controlling insulin secretion.

TRPM3 activation has frequently been monitored using either intracellular Ca^{2+} measurement with Ca^{2+} indicators and/or whole-cell patch-clamp to measure cationic membrane current (Wagner et al., 2008; Majeed et al., 2010). Thus, the influx of Ca^{2+} ions into the cells and the subsequent rise in the intracellular Ca^{2+} concentration was used as an indication for an activated TRPM3 channel. Recent data from our laboratory showed that pregnenolone sulfate-induced stimulation of endogenous TRPM3 channels in insulinoma cells activates a signal cascade that leads to an upregulation of gene transcription (Mayer et al., 2011; Müller et al.,

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2011). Pregnenolone sulfate also activates gene transcription in HEK293 engineered to express TRPM3 channels (Lesch et al., 2014).

Recently, we showed that stimulation of TRPM3 channels with pregnenolone sulfate upregulates activator protein (AP)-1 regulated gene transcription in insulinoma cells (Müller et al., 2011). Moreover, TRPM3 stimulation enhances expression of c-Fos and c-Jun, frequent constituents of the AP-1 transcriptional complex, in this cell type as well as in pancreatic islets in primary short-term culture (Müller et al., 2011). Here, we analyzed the signaling pathway leading to increased transcription of an AP-1-responsive reporter gene following stimulation of TRPM3 channels. Originally, AP-1 was described as a heterodimer of c-Jun and c-Fos (Chiu et al., 1988; Curran and Franza, 1988). These basic region leucine zipper (bZIP) transcription factors dimerize via their leucine zipper domains, which in turn bring together their basic domains to bind DNA in a sequence-specific manner. The current view is that AP-1 is actually a group of several distinct homodimers or heterodimers composed of various members of the Fos, Jun and ATF bZIP subfamilies. AP-1 functions in cells as a convergence point for many intracellular signaling cascades, initiated by cytokines, growth factors, hormones, and stressors such as UV light. The biological functions of AP-1 are tissue-specific and encompass the regulation of proliferation, transformation, differentiation, and programmed cell death (Shaulian and Karin, 2002). The results of this study show that TRPM3-mediated activation of AP-1 requires the influx of Ca^{2+} ions into the cells and arise in the intracellular Ca^{2+} concentration. The activation of MAP kinases connects the TRPM3-triggered cytosolic signaling cascade with changes of the gene expression pattern. In the nucleus, the bZIP proteins c-Jun and ATF2 and ternary complex factors (TCFs) are essential for TRPM3-induced upregulation of AP-1 controlled gene transcription. Thus, these data connect TRPM3 signaling with Ca^{2+} regulated transcription of c-Jun, ATF2 and TCF regulated genes.

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Materials and Methods

Cell culture

HEK293 cells containing the human TRPM3 coding region under the control of a tetracycline-regulated promoter were kindly provided by David Beech and Yasser Majeed, University of Leeds, UK and cultured as described (Naylor et al., 2008). TRPM3 expression was induced by adding tetracycline (1 μg/ml, Sigma-Aldrich # T7680, dissolved in water) to the culture medium containing 0.05 % fetal bovine serum for 24 hours prior to the stimulation with pregnenolone sulfate. Stimulation with pregnenolone sulfate (20 μM, Sigma # P162, dissolved in DMSO) was performed for 24 hours in DMEM containing 0.05 % fetal bovine serum.

Lentiviral gene transfer

The lentiviral transfer vectors pFUW-REST/Elk-1ΔC, pFUW-MEKK1Δ, pFUW-MKK6E, pFUWc-JunΔN, pFUW-ATF2ΔN, and pFUW-MKP-1, have been described previously (Mayer et al., 2008; Mayer and Thiel, 2009; Mayer et al., 2009; Rössler and Thiel, 2009; Müller et al., 2010; Spohn et al., 2010; Thiel et al., 2012). The GAL4 expression plasmid pFA2ATF2 was purchased from Stratagene. To generate a lentiviral vector encoding GAL4-ATF2, we digested plasmid pFA2ATF2 with ClaI, filled in with the Klenow fragment of DNA polymerase I, and recut with Ecl136II. The fragment was cloned into the HpaI site of the lentiviral transfer vector pFUW, thus generating the plasmid pFUW-GAL4-ATF2. The viral particles were produced as previously described (Keim et al., 2012a). In brief, viral particles were produced by transient transfection of 2.0 x 10^6 293T/17 cells using the calcium phosphate co-precipitation technique. Three plasmids were transfected into the cells plated on 60-mm plates: 6.6 μg of the transfer vector, 5 μg of the pCMVΔR8.91 packaging vector, and 2.3 μg of plasmid pCMVG, encoding the vesicular stomatitis virus glycoprotein. Transfections were performed in the presence of 25 μM chloroquine. Viral supernatants were harvested 60 hours after transfection, filtered through a 0.45 μm filter, and used to infect HEK293 cells in the presence of 8 μg/ml polybrene at 37^{o}C.

Lentiviral expression of short hairpin RNAs (shRNAs)

The lentiviral vector pLentiLox3.7 (pLL3.7) was purchased from American Type Culture Collection (Manassas, VA). The lentiviral transfer vectors pLLTRPM3, used to knock-down TRPM3 expression, and pLL3.7c-Jun, used to express a c-Jun-specific shRNA under the control of the U6 promoter, have been described (Mayer et al., 2011, Keim et al., 2012b). A

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lentiviral transfer vector (pLL3.7ATF2) expressing an ATF2-specific shRNA was designed as described (http://mcmanuslab.ucsf.edu/protocols/ll37stemloop_design.pdf), based on a published sequence (Bhoumik et al., 2002).

Reporter assays

The lentiviral transfer vectors pFWColl.luc, pFWColl.lucΔTRE, and pFWUAS^{5}Sp1^{2}luc have been described elsewhere (Rössler et al., 2008; Müller et al., 2010; Ekici et al., 2012). Cell extracts were prepared using reporter lysis buffer (Promega, Mannheim, Germany) and analyzed for luciferase activities as described (Thiel et al., 2000). Luciferase activity was normalized to the protein concentration.

Microscopy

Phase contrast and GFP fluorescence microscopy was done with a Zeiss Axiovert 200M microscope eqipped with the respective filter.

Ca^{2+} transient acquisition and analysis

For ratiometric Fura-2-based Ca^{2+} measurements, 293 cells containing a tetracycline-regulated TRPM3 expression unit were treated with or without tetracycline (1 µg/ml) 24 hours prior the experiments. After loading with Fura-2/AM and/or BAPTA-AM (both from Life Technologies, Darmstadt, Germany), cells were bathed in Tyrode's solution (135 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 10 mM glucose, 2 mM $MgCl_2$, and 10 HEPES adjusted to pH 7.35), mounted on a microscope (uiMic, TILL Photonics GmbH, Gräfelfing, Germany) and imaged through a 20x oil-immersion objective (UPLSAPO 20x oil 0.85, Olympus, Tokyo, Japan). Excitation was performed at alternating wavelengths 340/380 nm (15 nm FWHM with a PolyChrome V, TILL Photonics, Germany) while the emission (>510 nm) was imaged by a CCD camera (Retiga-2000R, Qimaging, Surrey, Canada) with 2 fps (image size: 400 x 300 pixels). Pregnenolone sulfate was administered by a local solenoid-controlled gravity-driven perfusion system. Where mentioned, Ca^{2+} free extracellular Tyrode was generated by omitting additional Ca^{2+} and adding 5 mM EGTA. The acquired images were analyzed using custom written macros in ImageJ (W. Rasband, NIH, Bethesda, USA). Images were background corrected and region-of-interest (ROI) fluorescence over time data was collected and further processed into Fura-2 ratios in Igor (Wavemetrics, Lake Oswego, USA) that was also employed for measuring the amplitude of the resulting Ca^{2+} transients. The final statistical analysis was undertaken in Prism 6 software (GraphPad Software Inc., Fl/USA).

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Western blots

Whole cell extracts and nuclear extracts were prepared as described (Kaufmann and Thiel, 2002). Proteins were separated by SDS-PAGE, blotted and incubated with antibodies directed against c-Jun (Santa Cruz, Heidelberg, Germany, # sc-1694), or ATF2 (Santa Cruz, Heidelberg, Germany, # sc-6233). The antibody directed against histone deacetylase-1 (HDAC1) was used as a loading control as previously described (Spohn et al., 2010; Mayer et al., 2011). To detect FLAG-tagged proteins, we used the M2 monoclonal antibody directed against the FLAG epitope (Sigma-Aldrich, Steinheim, Germany, # F3165) at 1:3000 dilution. Immunoreactive bands were detected via enhanced chemiluminescence as described (Spohn et al., 2010; Mayer et al., 2011). Values are expressed as the mean +/- SD from 3 independent experiments (n=3).

Statistics

Statistical analysis were done by using the two-tailed student´s *t*-test. Data shown are mean +/- SD from two to four independent experiments performed in quadruplicate. Statistical probability is expressed as $\star P < 0.05$, $\star\star P < 0.01$, and $\star\star\star P < 0.001$. Values were considered significant when $P < 0.05$.

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Results

Recently, we showed that stimulation of TRPM3 channels with pregnenolone sulfate induces a signal cascade in insulinoma cells that leads to an activation of gene transcription (Mayer et al., 2011; Müller et al., 2011). Insulinoma cells also express L-type voltage-gated Ca^{2+} channels that are - in addition to TRPM3 - involved in the regulation of pregnenolone sulfate-induced gene expression (Mayer et al., 2011). In contrast, in HEK293 cells engineered to express TRPM3 channels, gene transcription is activated following stimulation of TRPM3 stimulation with pregnenolone sulfate independently of L-type voltage-gated Ca^{2+} channels (Lesch et al., 2014). Thus, TRPM3 functions in this heterologous system in the absence of L-type voltage-gated Ca^{2+} channels as a ligand-activated ionotropic receptor, leading to an influx of Ca^{2+} into the cells following activation (Wagner et al., 2008; Majeed et al., 2010). To elucidate the signaling pathway connecting TRPM3 stimulation with enhanced gene transcription of AP-1 responsive genes, we used this engineered HEK293 cell line, in which expression of TRPM3 is induced by adding tetracycline to the culture medium as shown previously (Lesch et al., 2004). We thus avoided interference between TRPM3 and L-type voltage-gated Ca^{2+} channel signaling.

Pregnenolone sulfate triggers an upregulation of AP-1-mediated gene transcription in HEK293 cells expressing TRPM3

To measure AP-1 regulated transcription, we used a collagenase promoter/luciferase reporter gene. The collagenase promoter contains an AP-1 binding site in the proximal promoter region, also known as 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-responsive element (TRE). We and others have frequently used this promoter to monitor AP-1 activity (Angel et al., 1987; Steinmüller et al., 2001; Vries et al., 2001; Müller et al., 2010; 2011; Thiel and Rössler, 2011; 2014; Thiel et al., 2012; Kaufmann et al., 2013). We used lentiviral gene transfer to integrate the collagenase promoter/luciferase reporter gene into the genome of the cells. By this means, we were sure that the reporter gene was embedded into a nucleosomal context. In contrast, transient transfections of plasmids may result in incompletely organized reporter genes in comparison to the cellular chromatin, having a prokaryotic gene organisation including a nonrestrictive transcriptional ground state. A schematic depiction of the integrated provirus encoding the collagenase promoter/luciferase reporter gene is depicted in Fig. 1A, including the sequence of the wild-type and the mutated TRE. HEK293 cells containing a tetracycline-responsive TRPM3 expression casette were infected with a

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lentivirus encoding the collagenase promoter/luciferase reporter gene. Cells were treated with tetracycline to induce TRPM3 expression. Cells were serum-starved for 24 hours and stimulated with pregnenolone sulfate for twenty-four hours. Fig. 1B (left bars) shows that pregnenolone sulfate stimulation of HEK293 cells expressing TRPM3 induced an upregulation of reporter gene transcription. The AP-1 binding site within the collagenase promoter encompasses the sequence 5´-TGAGTCA-3´. Mutation of the TRE to 5´-TGATAGT-3´ abolished the effect of TRPM3 activation upon AP-1 regulated gene transcription (Fig. 1B, right bars). This experiment indicates that the TRE function as pregnenolone sulfate-responsive element in HEK293 cells. Fig. 1C shows that pregnenolone sulfate-stimulated collagenase promoter/luciferase reporter gene transcription was attenuated in cells expressing an a short-hairpin (sh) RNA directed against TRPM3, indicating that TRPM3 is required for stimulus-transcription coupling. This experiment supplements previously published data that showed that the TRPM3 inhibitor mefenamic acid blocked pregnenolone sulfate-induced AP-1 activation in TRPM3-expressing HEK293 cells (Lesch et al., 2014).

Essential role of extracellular and intracellular Ca^{2+} ions in pregnenolone sulfate-induced stimulation of AP-1 in HEK293 cells expressing TRPM3 channels

TRPM3 channels function as Ca^{2+} permeable cation channels. The influx of Ca^{2+} ions into the cells and the subsequent rise in the intracellular Ca^{2+} concentration has been frequenly used as an indication for an activated TRPM3 channel. We confirmed that stimulation of HEK293 cells expressing TRPM3 with pregnenolone sulfate increased the intracellular Ca^{2+} concentration. Cells with induced TRPM3 expression (Tet+) or naïve cells (Tet-) were challenged with pregnenolone sulfate to activate TRPM3 mediated Ca^{2+} influx. Fig. 2A shows exemplified typical Fura2-ratio traces. Fig. 2B depicts the statistical comparison (black and grey colour, respectively). In TRPM3 expressing HEK293 cells, pregnenolone sulfate resulted in a substantially increased Ca^{2+} response (black traces and black/grey bars). Pre-incubation of the cells with BAPTA-AM to increase the intracellular Ca^{2+} puffer capacity significantly decreased the response to pregnenolone sulfate in TRPM3 expressing cells and totally abrogated the response in naïve cells (red traces in Fig. 2A and red bars in Fig. 2B for statistical comparison). We found a similar behaviour when diminishing extracellular Ca^{2+} by addition of EGTA (green traces in Fig. 2A and green bars in Fig. 2B for statistical comparison).

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Recently, we showed that stimulation of Egr-1 expression stimulation of TRPM3 channels in pregnenolone sulfate-treated insulinoma cells required a rise in intracellular Ca^{2+} (Mayer et al., 2011). Here, we assessed the role of extracellular and intracellular Ca^{2+} ions in pregnenolone sulfate-induced activation of AP-1-regulated gene transcription in HEK293 cells expressing a tetracycline-controlled TRPM3 expression unit. First, we tested whether extracellular Ca^{2+} is required to upregulate AP-1 in pregnenolone sulfate-stimulated HEK293 cells expressing TRPM3. Fig. 2C shows that the activation of AP-1 following pregnenolone sulfate stimulation was completely blocked when the cells were cultured in Ca^{2+}-free medium containing EGTA. Thus, an influx of Ca^{2+} ions into the cells is essential to connect pregnenolone sulfate stimulation with enhanced AP-1 activity. Second, we tested the requirement of elevated intracellular Ca^{2+} levels for TRPM3-induced activation of AP-1-regulated gene transcription. The pregnenolone sulfate-induced elevation of $[Ca^{2+}]_i$ was attenuated by the preincubation with BAPTA-AM. As a result, the upregulation of AP-1 was significantly reduced (Fig. 2D). Hence, an influx of Ca^{2+} ions into the cells via TRPM3 and the subsequent elevation of $[Ca^{2+}]_i$ is essential for the activation of AP-1 following stimulation of TRPM3 expressing HEK293 cells with pregnenolone sulfate.

Protein phosphatase MKP-1 attenuates pregnenolone sulfate-induced AP-1 activation in HEK293 cells expressing TRPM3

Recently, we showed that expression of MAP kinase phosphatase-1 (MKP-1) impaired pregnenolone sulfate-induced upregulation of Egr-1 expression in insulinoma cells (Mayer et al., 2011). MKP-1 dephosphorylates and inactivates the MAP kinases extracellular signal-regulated protein kinase (ERK), p38 and c-Jun N-terminal protein kinase (JNK) in the nucleus (Shapiro and Ahn, 1998; Slack et al., 2001) and thus is part of a negative feedback loop. Activation of MAP kinases increases AP-1 activity (Karin, 1995; Whitmarsh and Davis, 1996), while MKP-1 impairs AP-1 regulated gene transcription (Rössler et al., 2008; Rössler and Thiel, 2009; Mayer et al., 2011; Thiel and Rössler, 2011; 2014; Thiel et al., 2012; Kaufmann et al., 2013). Therefore, we tested whether overexpression of MKP-1 counteracts the pregnenolone sulfate-induced transcription of an AP-1-regulated reporter gene as well. Fig. 3 shows that AP-1-regulated transcription was significantly reduced in pregnenolone sulfate-stimulated TRPM3-expressing HEK293 cells that had been infected with a MKP-1 encoding lentivirus. These data indicate that activated nuclear MAP kinases are required within the signaling cascade connecting TRPM3 channels with AP-1 regulated transcription.

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c-Jun is required for the upregulation of AP-1-mediated gene transcription in HEK293 cells expressing activated TRPM3 channels

The AP-1 transcription factor was originally described as a heterodimer of c-Jun and c-Fos (Chiu et al., 1988). Recently, we showed that stimulation of TRPM3 channels with pregnenolone sulfate induces the biosynthesis of c-Jun in HEK293 cells (Lesch et al., 2014). To assess the role of c-Jun in the regulation of AP-1 activity by TRPM3, we chose a dominant-negative approach. The modular structure of c-Jun and the dominant-negative mutant of c-Jun, c-JunΔN, is depicted in Fig. 4A. c-JunΔN encompasses amino acid residues from amino acid 188 to amino acid 331 of c-Jun. The dominant-negative mutant fails to activate transcription because it lacks the transcriptional activation domain. Rather, the mutant inhibits DNA-binding of its wild-type bZIP counterpart by blocking the cognate sites for DNA-binding. The biological activity of c-JunΔN was demonstrated in HEK293 cells that expressed constitutively active mutants of either mitogen-activated protein kinase/extracellular signal regulated kinase kinase kinase-1 (MEKK1) or MAP kinase kinase-6 (MKK6), MEKK1Δ and MKK6E, respectively. AP-1 regulated gene transcription was stimulated in cells overexpressing either MEKK1Δ or MKK6E. However, in cells that additionally expressed c-JunΔN, the upregulation of AP-1 activity was completely blocked (Supplemental Figures S1A, S1B). c-JunΔN was expressed in HEK293 cells following infection with a recombinant lentivirus. Proteins derived from nuclear extracts of 293/TRPM3 cells, either "mock"-infected cells or cells infected with a lentivirus encoding c-JunΔN, were fractionated by SDS-PAGE. The mutant was identified by Western blot analysis using an antibody that recognized the FLAG epitope (Fig. 4B). Next, we assessed the impact of c-JunΔN on TRPM3-mediated stimulation of AP-1. The results show that expression of c-JunΔN attenuated AP-1-regulated gene transcription in pregnenolone sulfate-stimulated HEK293 cells that expressed TRPM3 channels (Fig. 4C), indicating that c-Jun or a c-Jun dimerization partner is involved in the upregulation of AP-1 activity in TRPM3 expressing HEK293 cells that had been stimulated with pregnenolone sulfate.

To confirm the previous results, we expressed a c-Jun-specific small hairpin (sh) RNA in TRPM3-expressing HEK293 cells using lentiviral gene transfer. As a control, cells were infected with a lentivirus that was generated using the lentiviral transfer vector pLL3.7 (mock). To demonstrate the biological activity of the c-Jun-specific shRNA, HEK293 cells were either mock infected or infected with lentivirus encoding shRNAs directed against c-Jun. Cell extracts were prepared and analyzed for c-Jun immunoreactivity. In HEK293 cells

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expressing a c-Jun-specific shRNA, expression of c-Jun was significantly reduced (Fig. 4D), confirming previous data (Freund et al., 2004; Keim et al., 2012b). The provirus expressing the c-Jun-specific shRNA contained a second transcription unit that encoded EGFP under the control of the CMV promoter/enhancer. Expression of EGFP was used to measure the infection rate following lentiviral gene transfer. Supplemental Figure 2 shows that EGFP was expressed in almost all cells indicating a high rate of infection. Next, we assessed the effect of the c-Jun-specific shRNA on pregnenolone sulfate-induced stimulation of AP-1 in HEK293 cells expressing TRPM3. Fig. 4E shows that the upregulation of AP-1-regulated gene transcription by pregnenolone sulfate stimulation was almost completely blocked in the presence of a c-Jun-specific shRNA, indicating that c-Jun is required to connect the activation of the TRPM3 channels with enhanced AP-1 activity in the cells.

Stimulation of TRPM3 channels with pregnenolone sulfate induces an upregulation of the transcriptional activation potential of the bZIP protein ATF2

Activating transcription factor 2 (ATF2) is a bZIP protein that constitutes, together with bZIP proteins of the Fos and Jun families, the AP-1 transcription factor complex. We therefore asked whether ATF2 is also involved in the signaling cascade connecting TRPM3 stimulation with AP-1-regulated gene transcription. First, we assessed the transcriptional activation potential of ATF2, using a GAL4-ATF2 fusion protein that contained the phosphorylation-dependent activation domain of ATF2 (amino acids 1-96) and the DNA binding domain of the yeast transcription factor GAL4 (Fig. 5A). Since GAL4 does not bind to any known mammalian gene promoter element, interference by other transcriptional regulatory proteins was avoided. To measure the biological activities of the GAL4-ATF2 fusion protein we implanted a GAL4 responsive reporter gene into the chromatin of the cells to ensure that the reporter gene is packed into an ordered nucleosomal structure. Fig. 5B shows a schematic depiction of the integrated provirus, encoding the GAL4-responsive luciferase reporter gene. HEK293 cells containing a tetracycline-inducible TRPM3 expression unit, were infected with a lentivirus encoding the GAL4-responsive luciferase reporter together with a lentivirus that encoded for GAL4-ATF2. The results, depicted in Fig. 5C, revealed that the transcriptional activation potential of ATF2 was significantly elevated in TRPM3 expressing HEK293 cells that had been stimulated with pregnenolone sulfate.

ATF2 is required for the upregulation of AP-1-mediated gene transcription in HEK293 cells expressing activated TRPM3 channels

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We assessed the role of ATF2 in the regulation of AP-1 activity in HEK293 cells expressing activated TRPM3 channels using a dominant-negative mutant of ATF2, ATF2ΔN. The modular structure of the mutant is depicted in Fig. 6A. ATF2ΔN lacks the N-terminal regulatory regions and transcriptional activation domains, but contains ATF2 residues from amino acid 138 to amino acid 389, including the bZIP domain. Expression of ATF2ΔN was verified in HEK293 cells containing a tetracycline-responsive TRPM3 transcription unit, that were infected with an ATF2ΔN encoding lentivirus (Fig. 6B). To demonstrate the biological activity of the ATF2 mutant, we showed that expression of ATF2ΔN attenuated the stimulation of AP-1 in cells expressing constitutively active mutants of either MEKK1 or MKK6 (Supplemental Figures. S1A, S1B). Next, we assessed the role of ATF2 in the TRPM3-induced signaling cascade. Fig. 6C shows that expression of ATF2ΔN significantly interfered with the upregulation of AP-1-mediated gene transcription in HEK293 cells expressing stimulated TRPM3 channels.

These results were corroborated by expressing an ATF2-specific shRNA. Fig. 6D shows that the ATF2 levels were significantly reduced in HEK293 cells expressing this shRNA. The infection rate was controlled by assessing the expression of EGFP in HEK293 cells infected with a lentivirus that expressed the ATF2-specific shRNA (Supplemental Figure S2). To test the impact of ATF2 on the TRPM3-triggered signaling cascade, we expressed the ATF2-specific shRNA in TRPM3-expressing HEK293 cells using lentiviral gene transfer. As a control, cells were infected with a lentivirus that was generated with the lentiviral transfer vector pLL3.7 (mock). Fig. 6E shows that the upregulation of AP-1-regulated gene transcription by pregnenolone sulfate stimulation was significantly reduced in the presence of the ATF2-specific shRNA, indicating that ATF2 is part of the signaling pathway that connects the activation of the TRPM3 channels with enhanced AP-1 activity in the cells.

Suppression of ternary complex factor activity blocks the activation of AP-1 in pregnenolone sulfate-stimulated HEK293 cells expressing TRPM3

Expression of a dominant-negative mutant of the ternary complex factor Elk-1 attenuated the upregulation of the transcription factor Egr-1 following stimulation of insulinoma cells with pregnenolone sulfate (Mayer et al., 2011), indicating that Elk-1 or related ternary complex factors connect the transcription of the Egr-1 gene with the pregnenolone sulfate-induced intracellular signaling cascade elicited by the initial influx of Ca^{2+}. Recently, we showed that pregnenolone sulfate robustly enhanced the transcriptional activation potential of Elk-1 in

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HEK293 cells expressing a tetracycline-inducible TRPM3 expression unit (Lesch et al., 2014). TCFs are transcription factors that contact DNA and also bind to a dimer of the serum response factor (SRF), thus gerating a ternary complex. TCFs connect intracellular signaling cascades with transcriptional activation of serum response elements (SRE)-responsive genes (Cahill et al., 1995; Shaw and Saxton, 2003). In addition, we showed that TCFs are regulators of AP-1, most likely via controlling c-Fos expression (Müller et al., 2010; Thiel and Rössler, 2011; 2014; Thiel et al., 2012; Kaufmann et al., 2013). Therefore, we assessed whether TCFs also regulates AP-1 activity following stimulation of TRPM3 with pregnenolone sulfate. We expressed a dominant-negative mutant of the TCF Elk-1, termed REST/Elk-1ΔC, to overcome the problem associated with redundancy of functions between ternary complex factors (Cesari et al., 2004). The mutant retains the DNA binding and serum response factor (SRF) interaction domains, but lacks the C-terminal activation domain of Elk-1. REST/Elk-1ΔC additionally contains the N-terminal repression domain of the transcriptional repressor REST, a FLAG epitope for immunological detection and a nuclear localization signal (NLS). The modular structure of Elk-1 and REST/Elk-1ΔC is depicted in Fig. 7A. Expression of the Elk-1 mutant was verified by infecting HEK293 cells with a REST/Elk-1ΔC encoding lentivirus. The protein was identified by Western blot analysis using antibodies targeting the FLAG epitope (Fig. 7B). Fig. 7C shows that expression of REST/Elk-1ΔC almost completely prevented activation of AP-1 regulated gene transcription in TRPM3 expressing HEK293 cells that had been stimulated with pregnenolone sulfate. We conclude that TCF activation is essential for connecting TRPM3 activation with transcription of AP-1 controlled genes.

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Discussion

The steroid pregnenolone sulfate has been identified as a ligand for TRPM3 channels, leading to an influx of Ca^{2+} ions into pancreatic β-cells that express endogenous TRPM3 channels, or in HEK293 cells engineered to express TRPM3 (Wagner et al., 2008). Recently, we showed that pregnenolone sulfate regulates gene transcription in insulinoma cells and pancreatic islets. In particular, we showed that pregnenolone sulfate stimulation triggers the expression of the zinc finger transcription factor Egr-1 and the transcription of Egr-1-responsive target genes (Mayer et al., 2011). Furthermore, we showed that transcription of AP-1 and CREB-regulated genes are also enhanced following stimulation of TRPM3 with pregnenolone sulfate (Müller et al., 2011). The objective of this study was to investigate the intracellular signaling cascade connecting TRPM3 stimulation with an upregulation of AP-1 regulated gene transcription.

Experiments performed with insulinoma cells revealed that both TRPM3 channels and L-type voltage-dependent Ca^{2+} channels are required to induce gene transcription following application of pregnenolone sulfate to the cells (Mayer et al., 2011; Müller et al., 2011). Based on these data, we proposed that stimulation of TRPM3 with pregnenolone sulfate induces a depolarization of the plasma membrane of insulinoma cells that triggers the activation of L-type voltage-gated Ca^{2+} channels. As a result, a further influx of Ca^{2+} into the cells occurs that initiates an intracellular signaling cascade leading to changes in the gene expression pattern of the cells. In contrast to pancreatic β-cells, pregnenolone sulfate stimulation activates AP-1 and Egr-1-regulated gene transcription independently of L-type voltage-gated Ca^{2+} channels in HEK293 cells that do not express L-type voltage-gated Ca^{2+} channels and that were engineered to express TRPM3 (Lesch et al., 2014). Given the fact that stimulation of L-type voltage-gated Ca^{2+} channels activates c-Jun (Cruzalegui et al., 1999), a major constituent of the AP-1 transcriptional complex, we have used in this study the TRPM3-expressing HEK293 cells as cellular model to investigate TRPM3-induced AP-1 activation, avoiding interference between TRPM3 and L-type voltage-gated Ca^{2+} channels.

Using pharmacological tools, we have shown in this study that the influx of Ca^{2+} ions and the subsequent rise of the intracellular Ca^{2+} concentration is essential to activate AP-1-regulated gene transcription in TRPM3-expressing HEK293 cells that had been stimulated with pregnenolone sulfate. Elevation of the intracellular Ca^{2+} concentration is known to activate

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PKC, and subsequently the ERK signaling pathway (Schönwasser et al., 1998; Mayer and Thiel; 2009; Mayer et al., 2011). Accordingly, pharmacological inhibition of PKC with the compound bisindolylmaleimide III blocked the signaling cascade that connected TRPM3 channel activation with AP-1-regulated gene transcription. However, as bisindolylmaleimide III also inhibits the activity of other protein kinases, a final proof that PKC connects activated TRPM3 channels with the activation of the MAP kinase signaling cascade is still lacking.

AP-1 activity is regulated by MAP kinases in different cell types (Karin, 1995; Whitmarsh and Davis, 1996). Control experiments, depicted as supplement figures in this study, corroborated previous observations that activation of JNK and p38 results in enhanced AP-1 activity. In addition, AP-1 is activated following stimulation of the ERK signaling pathway (Kaufmann et al., 2013). Thus, overexpression of MKP-1, an enzyme that dephosphorylates and inactivates MAP kinases in the nucleus, attenuated pregnenolone sulfate-induced AP-1 activation. These data suggest that the phosphatase MKP-1 functions as nuclear shut-off devices that interrupt the signaling cascades induced by pregnenolone sulfate stimulation. Furthermore, these experiments indicate that the nuclear translocation of phosphorylated MAP kinases are required for TRPM3-regulated gene transcription. In insulinoma cells, activation of ERK is essential to trigger Egr-1 biosynthesis and an upregulation of Egr-1 activity following stimulation with pregnenolone sulfate (Mayer et al., 2011). Moreover, pregnenolone sulfate stimulation induces a sustained activation of ERK2 in the hippocampus (Chen et al., 2010). Expression of MKP-5, a nuclear phosphatase that dephosphorylates the protein kinases JNK and p38, reduced pregnenolone sulfate-triggered AP-1 activation (data not shown), suggesting that JNK, p38 and ERK are involved in connecting TRPM3 signaling with AP-1 activation. Future work will elucidate the role of these MAP kinases and the role of various PKC isoforms in the upregulation of AP-1 following stimulation of TRPM3 channels.

Initially described as a heterodimer of c-Jun and c-Fos, the today´s view is that the AP-1 transcriptional complex may be composed of several distinct homodimers or heterodimers of various members of the Fos, Jun and ATF bZIP subfamilies. The data described in this study show that the bZIP proteins c-Jun and ATF2 are involved in the regulation of AP-1 in HEK293 cells expressing activated TRPM3 channels. Given the fact that activation of TRPM3 triggers an influx of Ca^{2+} ions into the cells, these results implicate c-Jun and ATF2 as belonging to the Ca^{2+} responsive transcription factors – together with CREB, Egr-1, c-Fos and others (Sheng et al., 1991; Thiel et al., 2010). c-Jun has been described as a Ca^{2+}-regulated transcriptional activator in AtT20 cells following activation of L-type voltage gated

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Ca^{2+} channels (Cruzalegui et al., 1999). Interestingly, in this cellular system, Ca^{2+} mediated activation of c-Jun is independent of MAP kinase activation and relies on the activation of Ca^{2+}/calmodulin-dependent protein kinases. In pituitary gonadotrophs, c-Jun and ATF2 are phosphorylated and activated in response to stimulation of Gαq-coupled gonadotropin-releasing hormone receptors, involving elevated cytosolic Ca^{2+} levels, and activation of ERK and JNK (Mulvaney and Roberson, 2000; Xie et al., 2005; Mayer et al., 2008). Thus, elevation of the intracellular Ca^{2+} concentration, either via stimulation of TRPM3 ion channels or via activation of Gαq-coupled receptors that triggers an influx of Ca^{2+} from the ER into the cytosol, may be sufficient to activate c-Jun and ATF2.

The AP-1 transcriptional complex often includes the bZIP protein c-Fos. Recently, we showed that stimulation of TRPM3 channels in HEK293 cells with pregnenolone sulfate induces the biosynthesis of c-Fos (Lesch et al., 2014). Transcription of c-Fos is controlled by extracellular signaling molecules that target different trancription factors bound to the c-Fos promoter. Stimulation of insulinoma cells with pregnenolone sulfate activates TCFs such as Elk-1 that regulate, together with a dimer of the serum response factor (SRF), serum response element (SRE)-mediated transcription. Elk-1, a member of the Ets family of transcription factors, is a major nuclear substrate for the MAP kinases ERK, JNK and p38 and is an essential component of the ternary complex that binds to DNA and to a dimer of SRF. In fact, the TCFs as part of the SRE binding protein complex integrate MAP kinase signaling pathways, resulting in a change of the gene expression pattern (Cavigelli et al., 1995; Whitmarsh et al., 1995; Shaw and Saxton, 2003). Recently, we showed that the transcriptional activation potential of Elk-1 is upregulated in HEK293 cells expressing activated TRPM3 channels (Lesch et al., 2014). The c-Fos promoter contains a SRE and the molecular biology of SRE-mediated gene transcription has been elucidated in the analysis of the c-Fos gene (Cahill et al., 1995; Treisman, 1995). In this study, we proved the necessity of TCF activation within the TRPM3-induced signaling cascade by using a dominant negative version of Elk-1 in loss-of-function experiments. These experiments revealed that TCF activation is essential to connect TRPM3 stimulation with enhanced AP-1-mediated gene transcription in HEK293 cells.

In summary, this study shows the influx of Ca^{2+} ions via TRPM3 and activation of MAP kinases are integral parts of the signaling cascade connecting pregnenolone sulfate stimulation with enhanced AP-1 activity. In the nucleus, AP-1 activation is controlled by c-Jun, ATF2 and TCFs following stimulation of TRPM3 expressing cells with pregnenolone sulfate. Thus,

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this study connects for the first time the TRPM3-induced signaling cascade with transcription of target genes controlled by c-Jun, ATF2, and TCF.

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Authorship Contributions

Participated in research design: Thiel, Lesch

Conduced experiments: Lesch, Xin

Contributed new reagents and analytical tools: Thiel, Lipp

Performed data analysis: Lesch, Lipp, Thiel

Wrote or contributed to the writing of the manuscript: Thiel, Lesch, Lipp

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FOOTNOTES:

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LEGENDS FOR FIGURES

FIGURE 1

Pregnenolone sulfate stimulates AP-1-regulated transcription in TRPM3-expressing HEK293 cells

(A) Schematic representation of the integrated provirus encoding a collagenase promoter/luciferase reporter gene (Coll.luc). The promoter was inserted upstream of the luciferase reporter gene. The location and sequence of the intact or mutated 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-responsive element (TRE) within the collagenase promoter is depicted. The U3 region of the 5´ LTR of the transfer vector is deleted. The locations of the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) and the HIV flap element are indicated. (B) HEK293 cells containing a tetracycline-inducible TRPM3 transcription unit were infected with a recombinant lentivirus encoding the collagenase promoter/luciferase reporter gene (Coll.luc) with either an intact (left bars) or mutated TRE (right bars). The cells were serum-starved for 24 hours in the presence of tetracycline (1 μg/ml) and then stimulated with pregnenolone sulfate (PregS, 20 μM) for 24 hours. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. Data shown are mean +/- SD of three experiments performed in quadruplicate (✶✶✶, $P < 0.001$). (C) HEK293 cells containing a tetracycline-inducible TRPM3 transcription unit were infected with a recombinant lentivirus encoding the collagenase promoter/luciferase reporter gene (Coll.luc). In addition, cells were infected with lentiviruses encoding for a TRPM3-specific shRNA. As a control, cells were infected with a lentivirus generated with the lentiviral transfer plasmid pLL3.7 (mock). The cells were serum-starved for 24 hours in the presence of tetracycline (1 μg/ml) and then stimulated with pregnenolone sulfate (PregS, 20 μM) for 24 hours. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. Data shown are mean +/- SD of three experiments performed in quadruplicate (✶✶✶, $P < 0.001$).

FIGURE 2

Pregnenolone sulfate-induced activation of AP-1 in TRPM3-expressing HEK293 cells requires Ca^{2+}

(A, B) TRPM3 expression increases the amplitude of Ca^{2+} responses to pregnenolone sulfate stimulation that was sensitive to both intracellular Ca^{2+} buffering and a decrease in extracellular Ca^{2+}. Cells were loaded with Fura-2 and TRPM3 expression was either induced

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(Tet+) or not indueced (Tet-) by addition of tetracycline 24 hours before the experiment. Typical fura2-ratio traces of individual cells with TRPM3 expression (solid thick lines) or without induction (dashed lines) are displayed (A). The statistical summary of a larger population of cells is depicted in (B). (C) HEK293 cells expressing TRPM3 were infected with a recombinant lentivirus encoding a collagenase promoter/luciferase reporter gene. The cells were serum-starved for 24 hours in either DMEM or in Ca^{2+}-free medium that has been supplemented with EGTA (0.5 mM). Cells were stimulated with pregnenolone sulfate (20 μM) for 24 hours. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. Data shown are mean +/- SD of three experiments performed in quadruplicate (✶✶✶, $P < 0.001$). (D) HEK293 cells expressing TRPM3 were infected with a recombinant lentivirus encoding a collagenase promoter/luciferase reporter gene. The cells were serum-starved for twenty-four hours in DMEM. Cells were pre-incubated with BAPTA-AM (20 μM) or vehicle for 3 hours. Cells were stimulated with pregnenolone sulfate (20 μM) for 24 hours in the presence or absence of BAPTA-AM. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. Data shown are mean +/- SD of four experiments performed in quadruplicate (✶✶, $P < 0.01$).

FIGURE 3

MAP kinases connect TRPM3 activation with stimulation of AP-1 in HEK293 cells expressing TRPM3

HEK293 cells containing a tetracycline-inducible TRPM3 transcription unit were infected with a recombinant lentivirus encoding the collagenase promoter/luciferase reporter gene (Coll.luc). Cells were infected with a lentivirus encoding MKP-1. The transgenes were expressed under the control of the human ubiquitin-C promoter. As a control, cells were infected with a lentivirus generated with the lentiviral transfer plasmid pFUW (mock). The cells were serum-starved for 24 hours in the presence of tetracycline (1 μg/ml) and then stimulated with pregnenolone sulfate (PregS, 20 μM) for 24 hours. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. Data shown are mean +/- SD of three experiments performed in quadruplicate (✶✶✶, $P < 0.001$).

FIGURE 4

The transcription factor c-Jun connects TRPM3 activation with enhanced AP-1-mediated gene transcription

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(A) Modular structure of c-Jun and the dominant-negative form c-JunΔN. The dominant-negative mutant encompasses amino acid residues 188 to 331 of c-Jun, retaining the basic region leucine zipper domain (bZIP) responsible for DNA binding and dimerization, but lacking the NH_2-terminal transcriptional activation domain. (B) Western blot analysis of HEK293 cells containing a tetracycline-inducible TRPM3 transcription unit infected with a lentivirus encoding c-JunΔN. As a control, mock-infected cells were analyzed. Western blots were probed with the antibody directed against the FLAG epitope. (C) HEK293 cells containing a tetracycline-inducible TRPM3 transcription unit were infected with a lentivirus encoding the collagenase promoter/luciferase reporter gene (Coll.luc). In addition, cells were infected with a lentivirus encoding the c-Jun mutant c-JunΔN. As a control cells were infected with lentiviral stocks prepared with the lentiviral transfer vector pFUW (mock). The cells were serum-starved for 24 hours in the presence of tetracycline (1 μg/ml) and then stimulated with pregnenolone sulfate (PregS, 20 μM) for 24 hours. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration Data shown are mean +/- SD of two experiments performed in quadruplicate (✶✶, $P < 0.01$). (D) HEK293 cells expressing a tetracycline-inducible TRPM3 transcription unit were infected with a lentivirus that encoded for a c-Jun-specific shRNA. As a control, cells were infected with lentivirus generated with the lentivial transfer vector pLL3.7 (mock). Cells were incubated for 3 days, nuclear extracts were prepared and subjected to Western blot analysis using antibodies directed against c-Jun. The antibody directed against HDAC1 was used as a loading control. (E) HEK293 cells containing a tetracycline-inducible TRPM3 transcription unit were infected with lentiviruses encoding the collagenase promoter/luciferase reporter gene (Coll.luc). In addition, cells were infected with a lentivirus encoding a c-Jun-specific shRNA. As a control, cells were infected with a lentivirus generated with the lentiviral transfer vector pLL3.7 (mock). The cells were serum-starved for 24 hours in the presence of tetracycline (1 μg/ml) and then stimulated with pregnenolone sulfate (PregS, 20 μM) for 24 hours. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. Data shown are mean +/- SD of three experiments performed in quadruplicate (✶✶✶, $P < 0.001$).

FIGURE 5

Upregulation of the transcriptional activation potential of ATF2 following stimulation of TRPM3

(A) Schematic representation of the modular structure of ATF2 and GAL4-ATF2. ATF2 contains a phosphorylation-responsive N-terminal activation domain, including the threonine

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residues 69 and 71, that are phosphorylated by the protein kinases p38 MAP kinase and JNK. The GAL4-ATF2 fusion protein contains the N-terminal DNA-binding domain of the yeast transcription factor GAL4 fused to the N-terminal activation domain of human ATF2 encompassing amino acids 1 to 96. (B) Schematic representation of the integrated provirus encoding a luciferase reporter gene under the control of the minimal promoter, consisting of two Sp1 binding sites, a TATA box and an initiator element. Upstream of the minimal promoter, five GAL4 binding sites (UAS, upstream activating sequence) were inserted. (C) HEK293 cells containing a tetracycline-inducible TRPM3 transcription unit were double-infected with a lentivirus encoding a GAL4-responsive luciferase reporter gene and a lentivirus encoding GAL4-ATF2. Cells were treated for 24 hours with tetracycline to induce TRPM3 expression. Then, cells were serum-starved for 24 hours and then stimulated with pregnenolone sulfate (20 μM) for 24 hours. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. Data shown are mean +/- SD of three experiments performed in quadruplicate (✶✶✶, $P < 0.001$).

FIGURE 6

The transcription factor ATF2 connects TRPM3 activation with enhanced AP-1-mediated gene transcription

(A) Modular structure of ATF2 of the rat and the dominant-negative form ATF2ΔN. (B) Western blot analysis of HEK293 cells containing a tetracycline-inducible TRPM3 transcription unit infected with a lentivirus encoding ATF2ΔN. As a control, mock-infected cells were analyzed. Western blots were probed with the antibody directed against the FLAG epitope. (C) HEK293 cells containing a tetracycline-inducible TRPM3 transcription unit were infected with lentiviruses encoding the collagenase promoter/luciferase reporter gene (Coll.luc). In addition, cells were infected with a lentivirus encoding ATF2ΔN. As a control cells were infected with lentiviral stocks prepared with the lentiviral transfer vector pFUW (mock). The cells were serum-starved for 24 hours in the presence of tetracycline (1 mg/ml) and then stimulated with pregnenolone sulfate (PregS, 20 mM) for 24 hours. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. Data shown are mean +/- SD of three experiments performed in quadruplicate (✶✶, $P < 0.01$). (D) HEK293 cells expressing a tetracycline-inducible TRPM3 transcription unit were infected with a lentivirus that encoded for an ATF2-specific shRNA. As a control, cells were infected with lentivirus generated with the lentivial transfer vector pLL3.7 (mock). Cells were incubated for 3 days, nuclear extracts were prepared and subjected to Western blot analysis using antibodies directed against ATF2 or HDAC1 (loading control).

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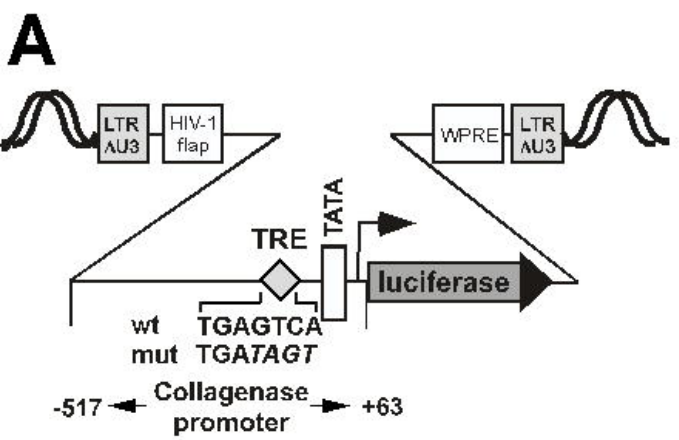
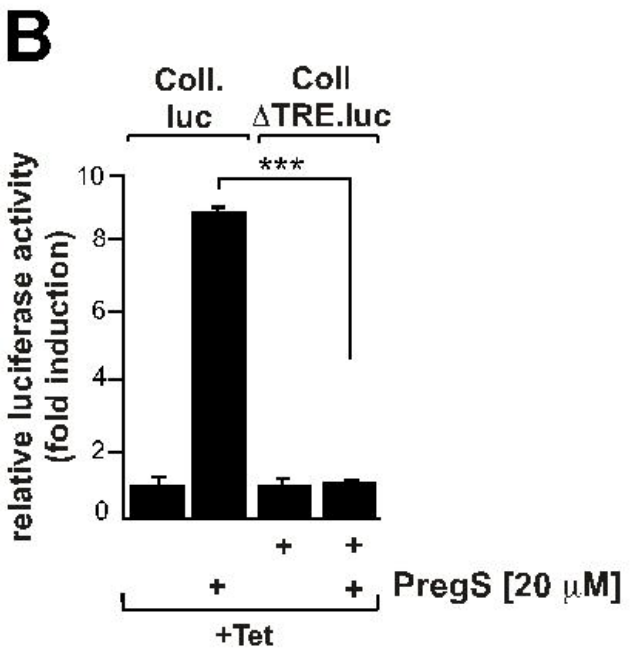
(E) HEK293 cells containing a tetracycline-inducible TRPM3 transcription unit were infected with lentiviruses encoding the collagenase promoter/luciferase reporter gene (Coll.luc). In addition, cells were infected with a lentivirus encoding an ATF2-specific shRNA. As a control, cells were infected with lentivirus generated with the lentivial transfer vector pLL3.7 (mock). The cells were serum-starved for 24 hours in the presence of tetracycline (1 mg/ml) and then stimulated with pregnenolone sulfate (PregS, 20 mM) for 24 hours. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. Data shown are mean +/- SD of three experiments performed in quadruplicate (✶✶, $P < 0.01$).

FIGURE 7

Essential role of ternary complex factors for controlling AP-1 activity in HEK293 cells expressing stimulated TRPM3 channels

(A) Schematic representation of the modular structure of Elk-1 and the dominant-negative mutant REST/Elk-1ΔC. (B) Western blot analysis of mock-infected HEK293 cells or cells infected with a recombinant lentivirus encoding REST/Elk-1ΔC. (C) Expression of REST/Elk-1ΔC blocks pregnenolone sulfate-induced upregulation of AP-1-mediated transcription in HEK293 cells expressing TRPM3. HEK293 cells containing a tetracycline-inducible TRPM3 transcription unit were infected with lentiviruses encoding the collagenase promoter/luciferase reporter gene (Coll.luc). In addition, cells were either mock-infected or infected with a recombinant lentivirus encoding REST/Elk-1ΔC. The cells were serum-starved for 24 hours in the presence of tetracycline (1 μg/ml) and then stimulated with pregnenolone sulfate (PregS, 20 μM) for 24 hours. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. Data shown are mean +/- SD of three experiments performed in quadruplicate (✶✶✶, $P < 0.001$).


A
LTR
ΔU3
HIV-1
flap
WPRE
LTR
ΔU3
TRE
TATA
luciferase
wt TGAGTCA
mut TGATATGT
-517
Collagenase
promoter
+63


B
Coll.
luc
Coll
ΔTRE.luc
***
relative luciferase activity
(fold induction)
10
8
6
4
2
0
+
+
+
+
PregS [20 μM]
+Tet

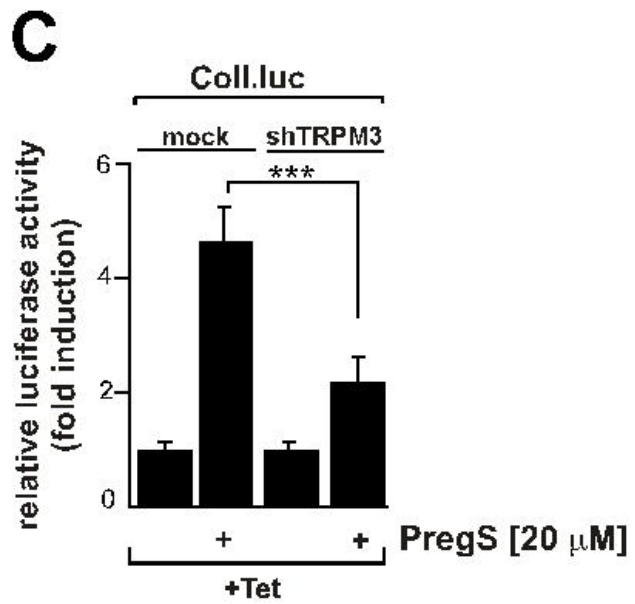

C
Coll.luc
mock
shTRPM3
***
relative luciferase activity
(fold induction)
6
4
2
0
+
+
PregS [20 μM]
+Tet

Fig. 1

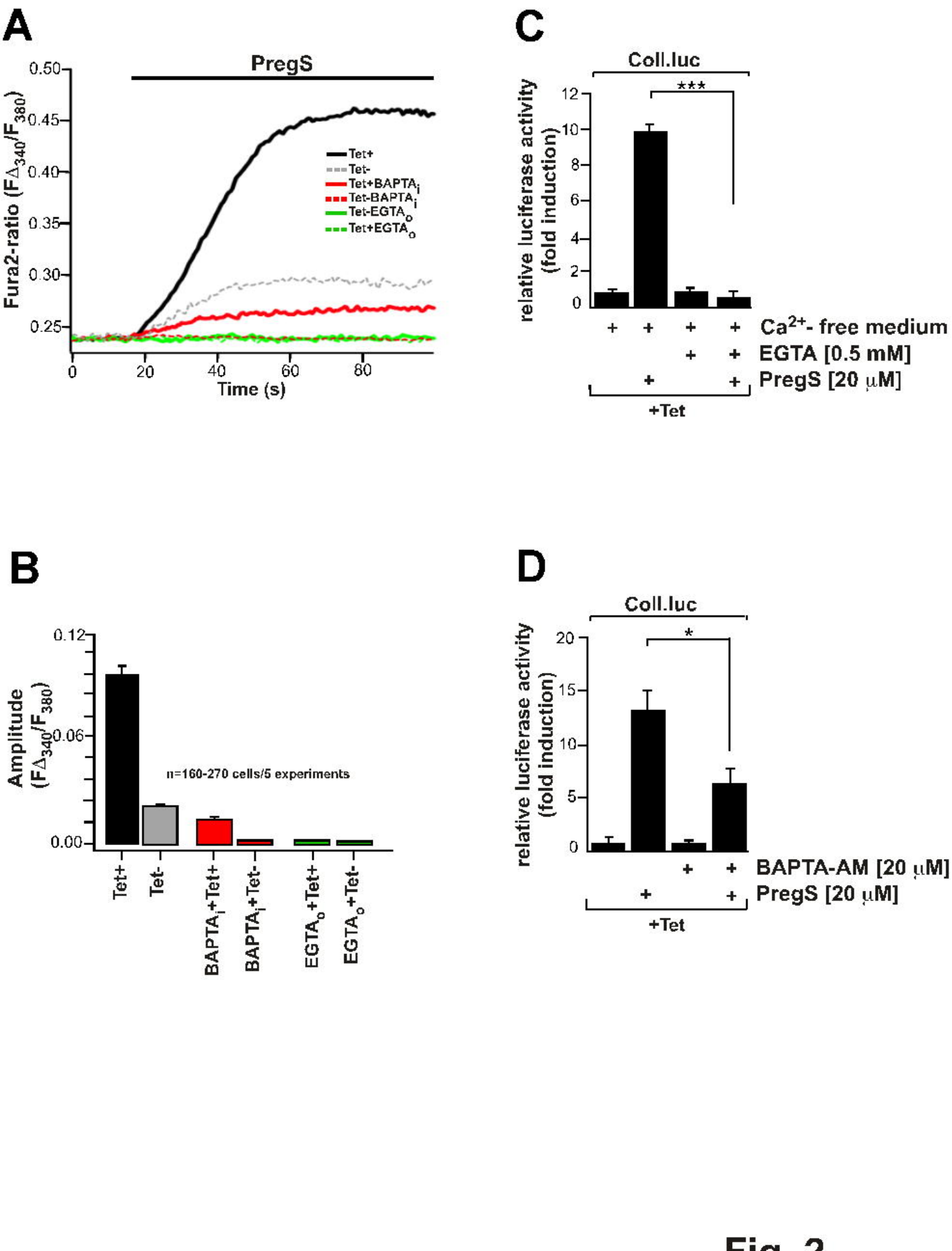

A
PregS
Fura2-ratio (FΔ340/F380)
0.50
0.45
0.40
0.35
0.30
0.25
0
20
40
60
80
Time (s)
Tet+
Tet-
Tet+BAPTAi
Tet-BAPTAi
Tet-EGTAo
Tet+EGTAo
B
Amplitude (FΔ340/F380)
0.12
0.06
0.00
n=160-270 cells/5 experiments
Tet+
Tet-
BAPTAi+Tet+
BAPTAi+Tet-
EGTAo+Tet+
EGTAo+Tet-
C
Coll.luc
relative luciferase activity (fold induction)
12
10
8
6
4
2
0
***
+ + + + Ca2+- free medium
+ + EGTA [0.5 mM]
+ + PregS [20 μM]
+Tet
D
Coll.luc
relative luciferase activity (fold induction)
20
15
10
5
0
*
+ + BAPTA-AM [20 μM]
+ + PregS [20 μM]
+Tet

Fig. 2

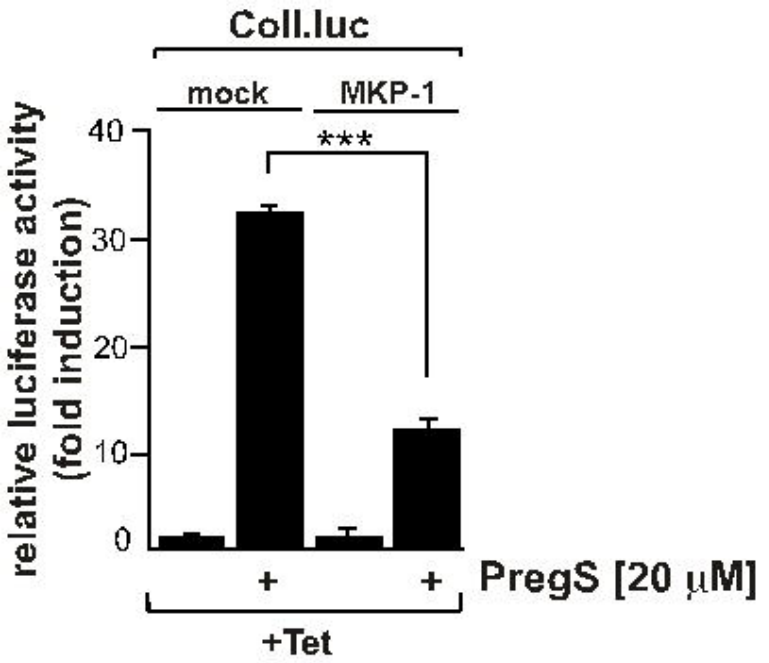

Coll.luc
mock
MKP-1
***
relative luciferase activity
(fold induction)
40
30
20
10
0
+
+
PregS [20 μM]
+Tet

Fig. 3

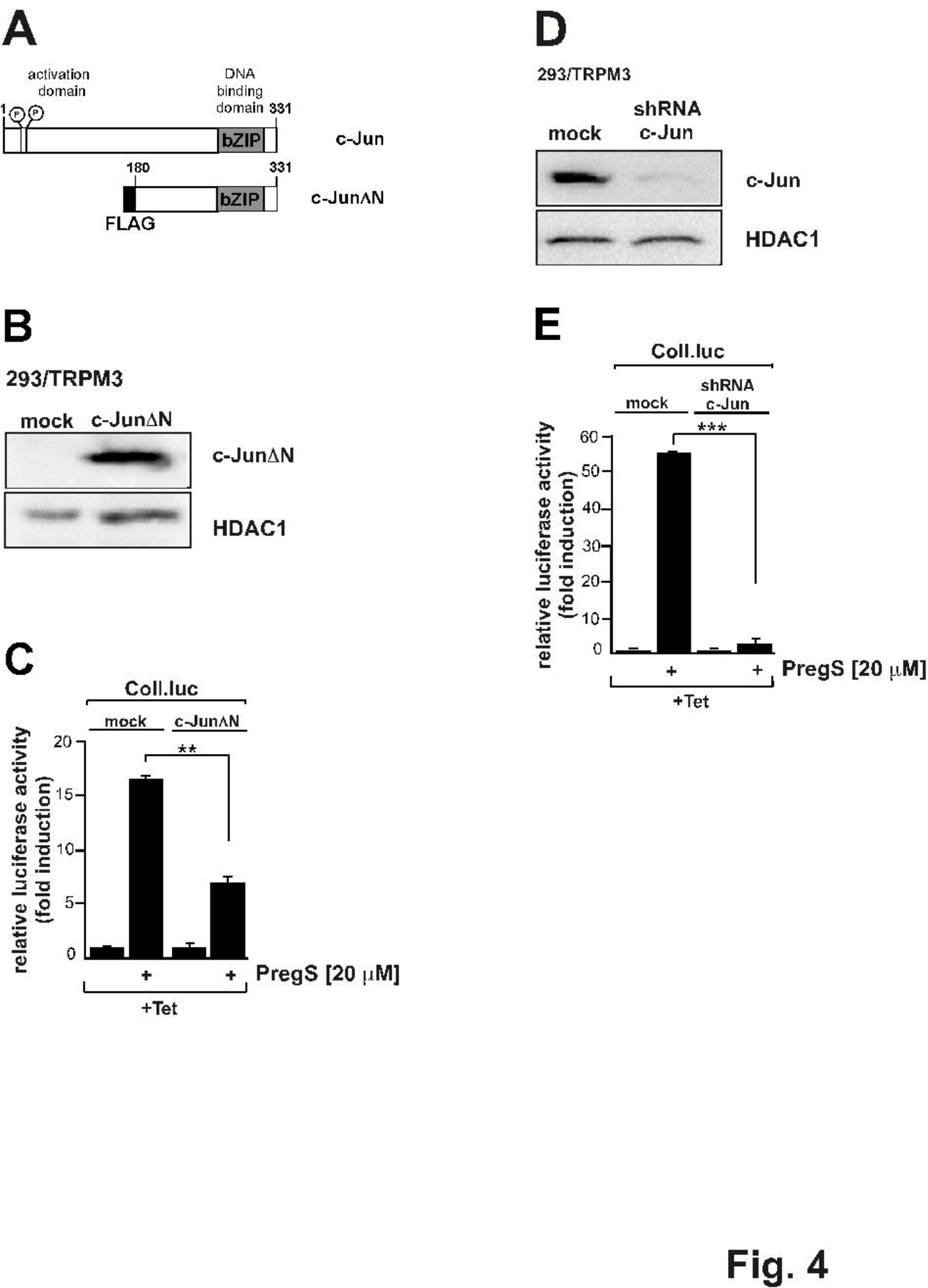
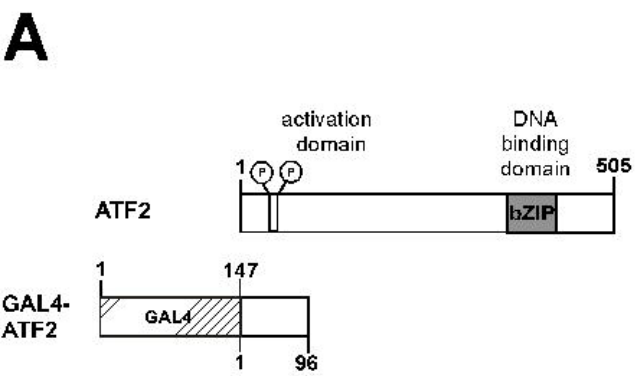
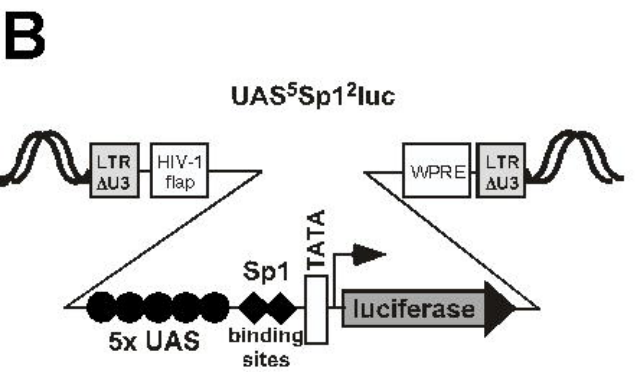

A
activation domain
DNA binding domain
1
331
bZIP
c-Jun
180
331
bZIP
c-JunΔN
FLAG
B
293/TRPM3
mock
c-JunΔN
c-JunΔN
HDAC1
C
Coll.luc
mock
c-JunΔN
**
relative luciferase activity (fold induction)
20
15
10
5
0
+
+
PregS [20 μM]
+Tet
D
293/TRPM3
shRNA
mock
c-Jun
c-Jun
HDAC1
E
Coll.luc
mock
shRNA c-Jun
***
relative luciferase activity (fold induction)
60
50
40
30
20
10
0
+
+
PregS [20 μM]
+Tet

Fig. 4


A
activation domain
DNA binding domain
1
P
P
505
ATF2
bZIP
1
147
GAL4-ATF2
GAL4
1
96


B
UAS5Sp12luc
LTR ΔU3
HIV-1 flap
WPRE
LTR ΔU3
TATA
Sp1
luciferase
5x UAS
binding sites

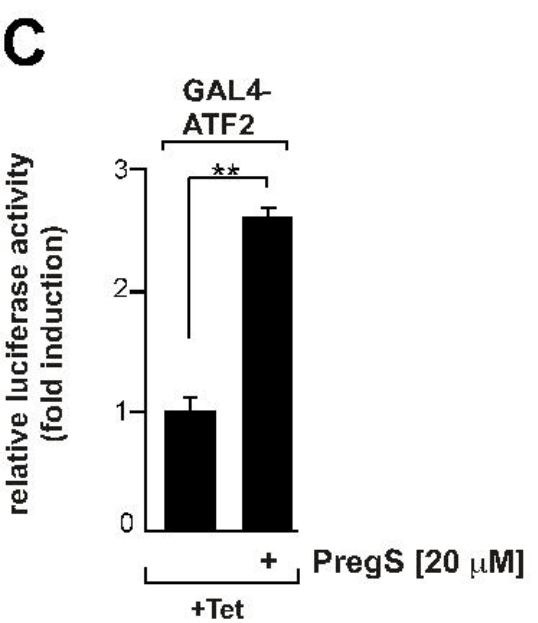

C
GAL4-ATF2
**
relative luciferase activity (fold induction)
3
2
1
0
+
PregS [20 μM]
+Tet

Fig. 5

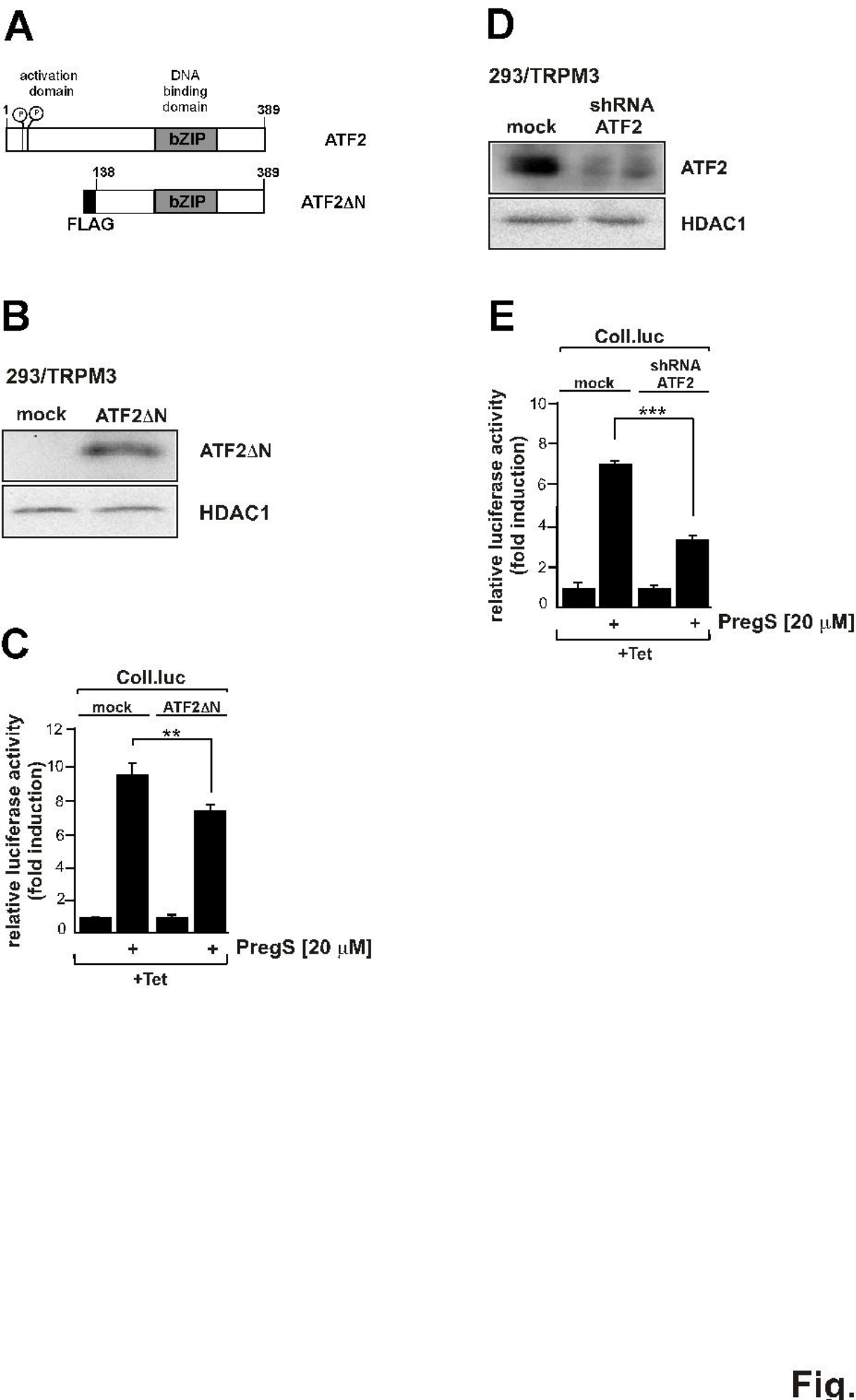

A
activation domain
DNA binding domain
1
P
P
389
bZIP
ATF2
138
389
bZIP
ATF2ΔN
FLAG
B
293/TRPM3
mock
ATF2ΔN
ATF2ΔN
HDAC1
C
Coll.luc
mock
ATF2ΔN
relative luciferase activity (fold induction)
12
10
8
6
4
2
0
**
+
+
PregS [20 μM]
+Tet
D
293/TRPM3
mock
shRNA ATF2
ATF2
HDAC1
E
Coll.luc
mock
shRNA ATF2
relative luciferase activity (fold induction)
10
8
6
4
2
0
***
+
+
PregS [20 μM]
+Tet

Fig. 6

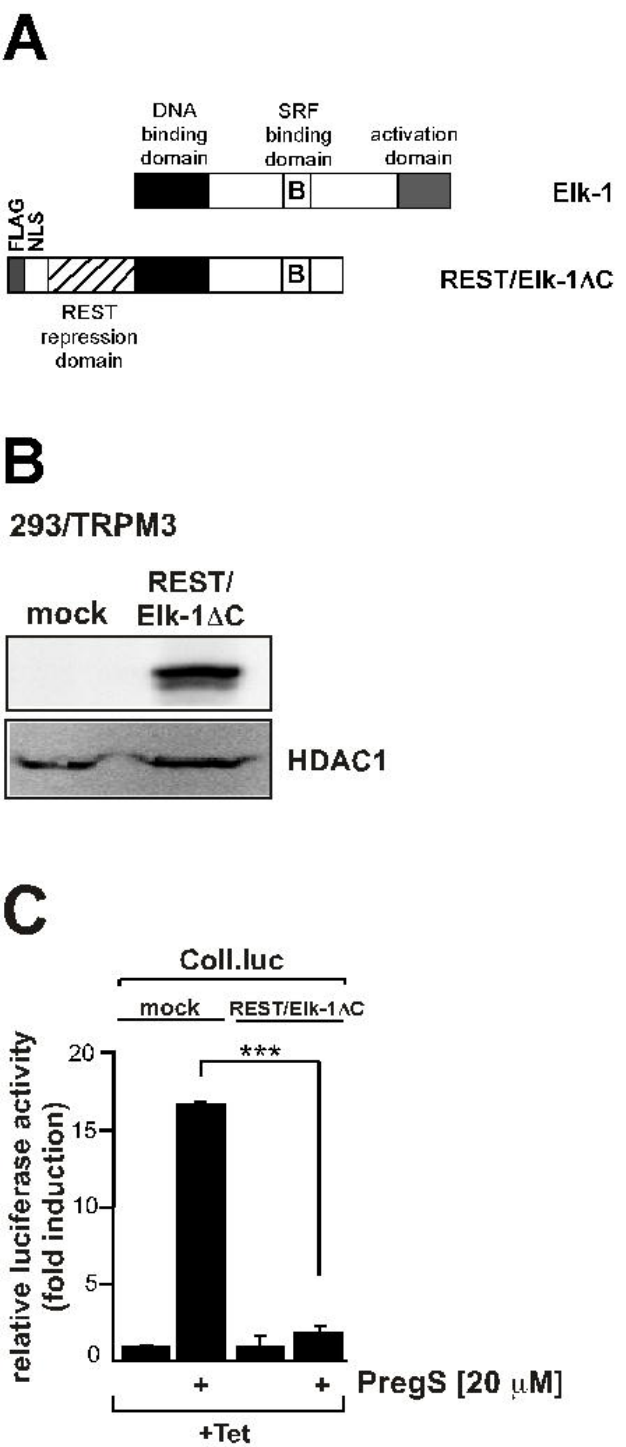

A
DNA binding domain
SRF binding domain
activation domain
B
Elk-1
FLAG NLS
B
REST/Elk-1ΔC
REST repression domain
B
293/TRPM3
mock
REST/ Elk-1ΔC
HDAC1
C
Coll.luc
mock
REST/Elk-1ΔC
***
relative luciferase activity (fold induction)
20
15
10
5
0
+
+
PregS [20 μM]
+Tet

Fig. 7